

Vaccination against Gonadotropin-releasing Hormone (GnRH) Using Toxin Receptor-binding Domain-conjugated GnRH Repeats¹

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Abstract

A method for the preparation of an immunogen containing multiple copies of a self-peptide in linear alignment was designed in order to overcome the difficulty of inducing an immune response to poorly immunogenic peptide antigens. DNA fragments encoding multiple repeats of the self-peptide were generated by a new technique, termed template-repeated polymerase chain reaction (TR-PCR), which could be subcloned into an expression vector for production of peptide repeats as an immunogen. This approach was tested by constructing fusion proteins containing the receptor-binding domain of *Pseudomonas* exotoxin A and multiple copies of the 10-residue sequence of the peptide hormone gonadotropin-releasing hormone (GnRH). Immunization of female rabbits with the immunogen that contained the exotoxin receptor-binding domain and 12 copies of GnRH (PEIa-GnRH₁₂) resulted in the generation of high-titer antibodies specific for GnRH. Although at equal molar basis of the GnRH moiety, the immunogen that contained single copy of GnRH (PEIa-GnRH₁) induced low-titer anti-GnRH antibodies. These observations suggest that the presence of multiple peptide repeats is a key factor in eliciting an immune response. In addition, anti-GnRH antibodies effectively neutralized GnRH activity *in vivo*, as demonstrated by the degeneration of the ovaries in the injected rabbits. Because anti-GnRH antibody could be functionally analogous to GnRH antagonist, which has been used to treat patients with ovarian cancer, vaccination of PEIa-GnRH₁₂ presents a potential therapeutic application for the treatment of GnRH-sensitive ovarian cancer.

Introduction

The induction of an immune response against specific self-peptides is potentially beneficial for the treatment of certain diseases. The results of such peptide-based immunization in animals, however, have not been satisfactory because of the low immunogenicity of self-peptides, a low efficiency of chemical conjugation, and the heterogeneous nature of antigen preparations. To address these problems, we have investigated the use of peptide repeats conjugated to the receptor-binding domain of *Pseudomonas* exotoxin A to induce antibodies that might suppress the function of overproduced self-proteins. Application of cell-binding toxin subunit as an adjuvant and antigen vector has been reported (1). In this study, the receptor binding domain of *Pseudomonas* exotoxin A was chosen because of its ability to deliver antigens into antigen-presenting cells through receptor-mediated endocytosis, which is more efficient than phagocytosis. As a target antigen for the development of a therapeutic vaccine, we chose GnRH.³

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³ The abbreviations used are: GnRH, gonadotropin-releasing hormone; TR-PCR, template-repeated PCR; Pfu, *Pyrococcus furiosus*; GST, glutathione S-transferase.

GnRH is an endogenous hormone that contains 10 amino acid residues (2). If an antigen construct containing linear repeats of GnRH is able to induce an autoimmune response, it might be expected that any linear self-peptide repeat would also prove immunogenic. Furthermore, GnRH functions as a key hormone in the regulation of the pituitary-gonadal axis and also likely affects various other tissues in addition to the pituitary (2–4). The expression of GnRH or its receptors is increased in several human malignancies, including cancer of the breast, ovary, endometrium, and prostate (4–6). The induction of an immune response to GnRH might thus prove beneficial in the treatment of such GnRH-associated diseases.

Materials and Methods

Materials. Heat stable *Pfu* DNA polymerase, GnRH, angiotensin I and pGEM-T Easy vector system were purchased from Promega. Ex. *Taq* polymerase was obtained from Takara Co. His-Bind Resin was from Novagen. Bacteria strains, *E. coli* XL10-Glod and BL21(DE3)*lysS*, used for DNA cloning and protein production were from Stratagene. Female New Zealand White rabbits were acquired from the animal center at National Taiwan University. [³H]GnRH was purchased from NEN. Reagents for ELISA and immunoblotting were from GIBCOBRL. Most other chemicals, unless specified, were from Sigma Chemical Co.

Construction of DNA Fragments Encoding GnRH Repeats and Immunogen-containing Toxin Receptor-binding Domain-conjugated GnRH Repeats. Ten pM of GnRH coded single strain DNA sequences Oligonucleotide A and Oligonucleotide B (as a half complement to Oligonucleotide A) were used as both primers and templates in the TR-PCR. The thermal cycle is 30 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 30 s, and polymerization at 72°C for 30 s, followed by a final polymerization step at 72°C for 10 min. The products of TR-PCR at 100-fold dilution were subjected to adapter-PCR with the adapter primers as shown in Fig. 1c. The amplification protocol was identical to that for TR-PCR, with the exception that the denaturation step was performed for 1 min. The products of adapter-PCR, which contained a *Sac*II site at the 5' end, and *Eco*RI site at the 3' end, and a stop codon at the end of the coding region, were then subcloned into a T Easy vector. Various repeats of GnRH clone were subcloned into plasmid pPEDI at the 3' end of the coding region for domain Ia, the receptor-binding domain, of *Pseudomonas* exotoxin A. The resulting plasmid, pPEDI_{Gn} (n for 1, 9, 12) was introduced into *E. coli* BL21(DE3) *lysS*, and the expressed fusion protein (PEIa-GnRH_n) was purified with affinity chromatography on a nickel-agarose column as described in Fig. 2b.

Animal Experiments. Sixteen-week-old female New Zealand White rabbits were injected subcutaneously with 0.1 to 1.0 mg of various antigens three times at biweekly intervals. Sera were collected weekly for immunoassay. Animals injected for three consecutive weeks were sacrificed at 26 weeks after the last injection. The ovaries were individually weighed for statistical analysis.

Immunocompetitive Assay. A trace amount of [³H]-GnRH (1 μCi, specific activity 54 Ci/mmol), 50 μl of protein A Sepharose-CL4B (0.05 mg protein A/ml), 7 μl antisera and various amounts of competitor (GnRH or angiotensin I) in PBS in a final volume of 0.5 ml were incubated at 37°C for 2 h, followed by centrifugation to remove unbound [³H]-GnRH. The pellets were washed three times with PBS prior to scintillation counting. [³H]-GnRH

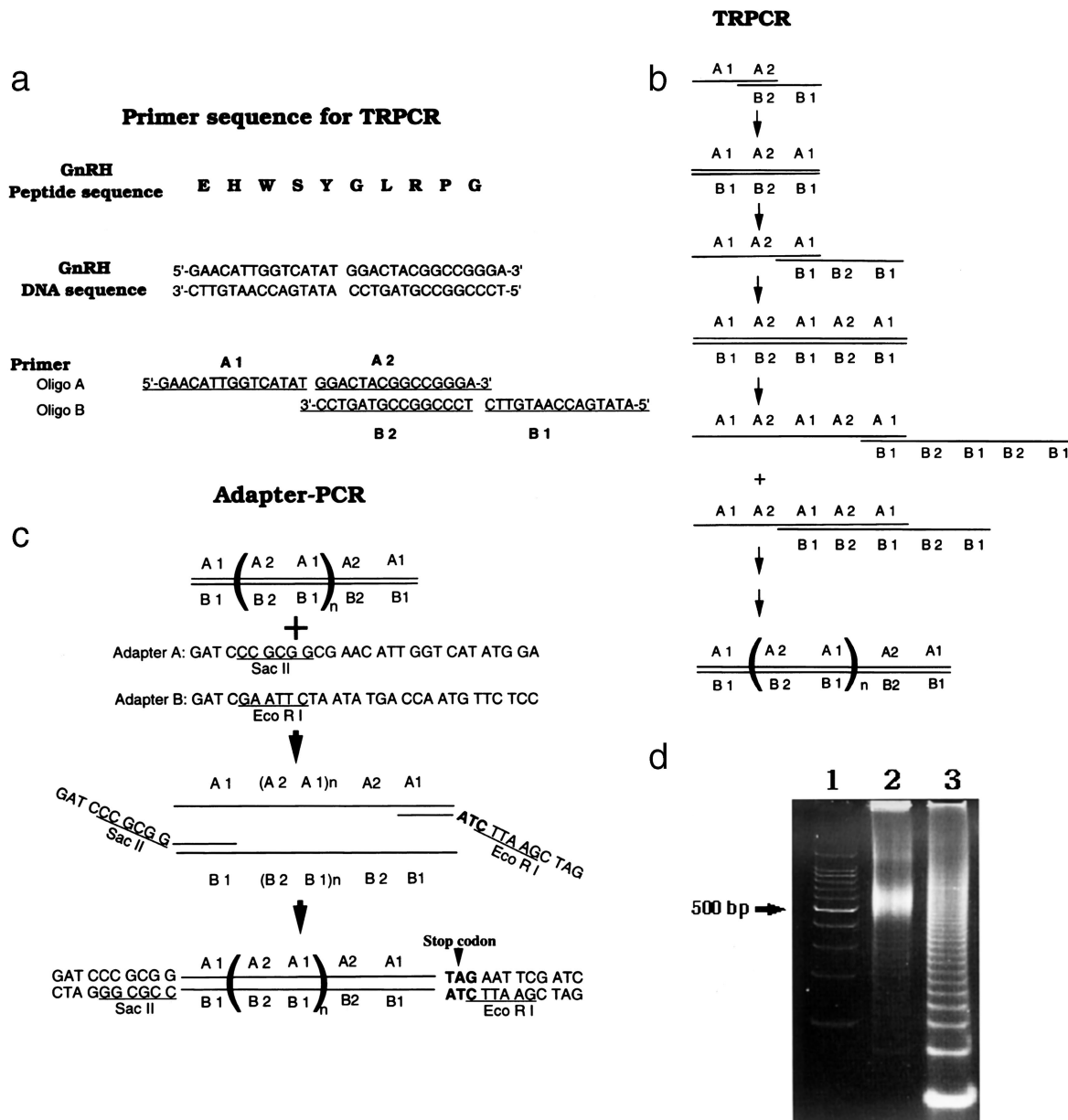


Fig. 1. The strategy for TR-PCR. *a*, oligonucleotide primers for TR-PCR. Oligo A encodes the target antigen (GnRH) and oligo B is complementary to oligo A in the manner indicated. Thus, the 5' half (A1) of oligo A is complementary to the 5' half (B1) of oligo B, and the 3' half of oligo A (A2) is complementary to the 3' half (B2) of oligo B. *b*, TR-PCR. Oligo A and oligo B were used as both primers and templates for TR-PCR, the protocol for which comprised 30 cycles of denaturation at 94°C for 30 s, annealing at 37°C for 30 s, and polymerization at 72°C for 30 s, followed by a final polymerization step at 72°C for 10 min. *c*, adapter-PCR. The products of TR-PCR (1:100 dilution) were subjected to adapter-PCR with the adapter primers A and B. The amplification protocol was identical to that for TR-PCR, with the exception that the denaturation step was performed for 1 min. The products of adapter-PCR, which contained a *Sac*II site at the 5' end, an *Eco*RI site at the 3' end, and a stop codon at the end of the coding region, were then subcloned into a T vector. *d*, analysis of the products of TR-PCR and adapter-PCR. The products of TR-PCR (Lane 2) and adapter-PCR (Lane 3) were analyzed by PAGE on an 8% gel and ethidium bromide staining. Lane 1, DNA size markers.

bound to antisera in the absence of competitor was used as a control, representing 100% of the [³H]-GnRH binding activity.

Results

To synthesize toxin-conjugated GnRH repeats, we developed a new technique termed TR-PCR (Fig. 1). We designed two oligonucleotides, oligo A and B. Oligo A encodes the target antigen (rabbit GnRH, which is identical in sequence to the human hormone), and oligo B exhibits overlapping complementarity to oligo A as shown in Fig. 1*a*. Because the TR-PCR products also serve as DNA templates for the generation of linear GnRH repeat sequences (Fig. 1*b*), we chose *Pfu* DNA polymerase instead of *Taq* polymerase to catalyze the

reaction in order to prevent insertion or misincorporation of bases during amplification (7). To incorporate restriction sites for subcloning at both ends of the TR-PCR products (*Sac*II at the 5' end and *Eco*RI at the 3' end) as well as a stop codon at the 3' end of the coding region, we performed a second PCR (adapter-PCR) with two adapter primers (Fig. 1*c*). The products of TR-PCR and adapter-PCR were examined by PAGE. Most of the TR-PCR products were between 500 and 700 bp in size, whereas the size distribution of the products of adapter-PCR showed a ladder-like pattern (Fig. 1*d*). The prominent lower band in this ladder corresponds to the GnRH DNA dimer, with the higher bands corresponding to the trimer, tetramer, and so forth; as the size of the products increased, the intensity decreased gradually.

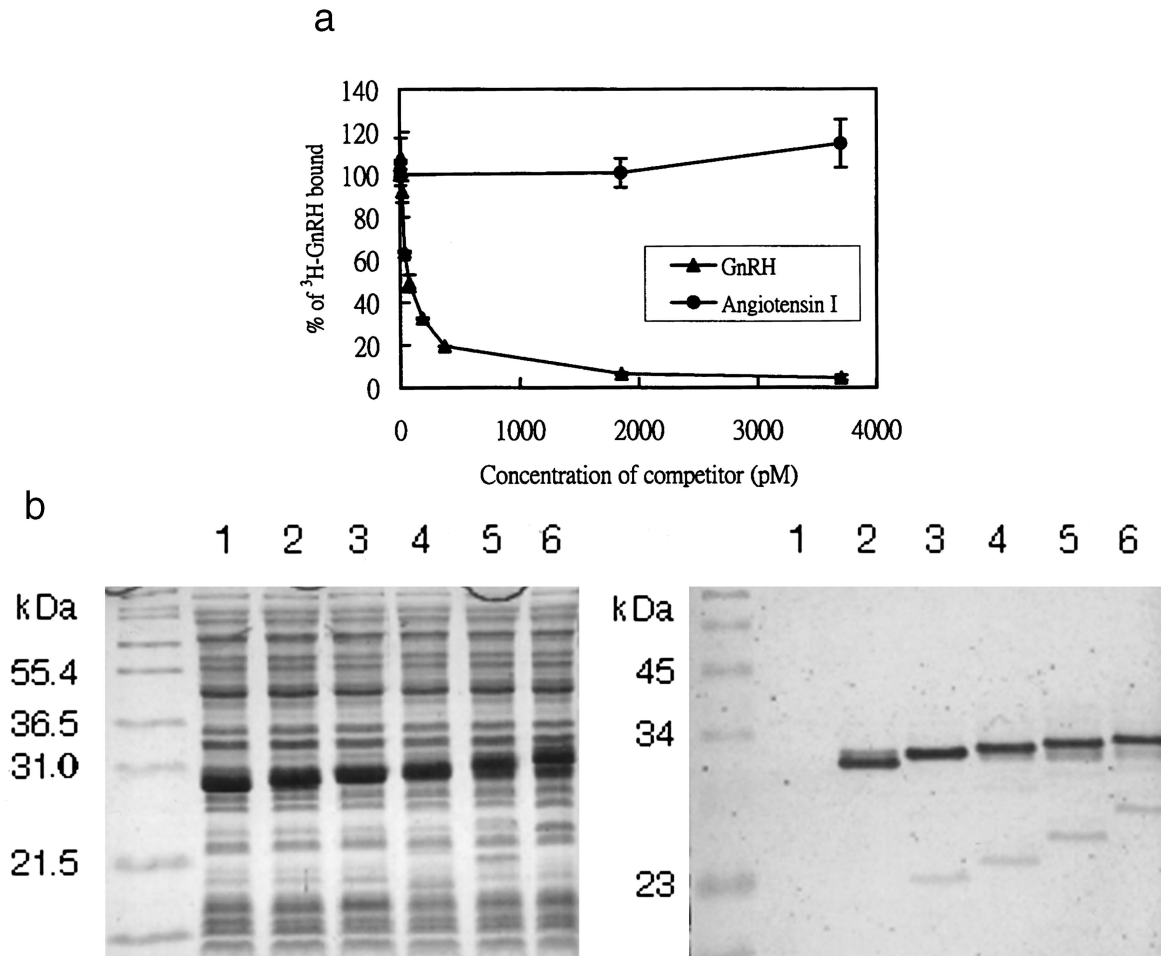


Fig. 2. Characterization of the antisera obtained from rabbits immunized with PEIa-GnRH₁₂. *a*, specificity analysis of the antisera. To examine whether the antibodies induced by PEIa-GnRH₁₂ reacted with native GnRH, an immunocompetitive assay was performed. A trace amount of [³H]GnRH (1 μ Ci; specific activity, 54 Ci/mmol), 50 μ l of protein A Sepharose-CL4B (0.05 mg of protein A/ml), 7 μ l of antisera, and various amounts of competitor (GnRH or angiotensin I) in PBS in a final volume of 0.5 ml were incubated at 37°C for 2 h, followed by centrifugation to remove unbound [³H]GnRH. The pellets were washed three times with PBS before scintillation counting. [³H]GnRH bound to antisera in the absence of competitor was used as a control, representing 100% of the [³H]GnRH binding activity. *b*, Epitope analysis of anti-GnRH antibodies. *Left*, test antigens for epitope analyses were prepared by subcloning DNA fragments encoding one to five copies of GnRH into plasmid pGEXKKG (16), and introducing the resulting constructs into *E. coli* BL21(DE3)/lysS. The transformants were cultured at 37°C in Luria Bertoni medium containing ampicillin (50 μ g/ml), chloramphenicol (25 μ g/ml), and tetracycline (10 μ g/ml). When the absorbance at 600 nm of the culture reached 0.2, isopropyl- β -D-thiogalactopyranoside was added to a final concentration of 0.1 mM, and the cells were cultured for an additional 90 min. Cells (20×10^6 per lane) were dissolved in Laemmli sample buffer, heated at 90°C for 5 min, and then analyzed by SDS-PAGE on a 15% gel and Coomassie Blue staining. *Lane 1*, GST; *Lane 2*, GST-GnRH; *Lane 3*, GST-GnRH2; *Lane 4*, GST-GnRH3; *Lane 5*, GST-GnRH4; and *Lane 6*, GST-GnRH5. The left-most lane contains molecular size standards. (*kDa*, molecular weight in thousands.) *Right*, serum collected from rabbits immunized with PEIa-GnRH₁₂ was analyzed for the epitopes of anti-GnRH antibodies. Bacterial cells (1×10^6 per lane) expressing the various test antigens were fractionated by SDS-PAGE, and the separated proteins were transferred to a polyvinylidene difluoride filter (Millipore) and subjected to immunoblot analysis with the rabbit antibodies, followed by incubation with antirabbit antibody conjugated with alkaline phosphatases, and then stained with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. Lanes are as in the left panel.

The adapter-PCR products were then subcloned into a T vector. We screened 24 positive clones and found that they contained from 3 to 12 GnRH DNA repeats. One clone containing 12 such repeats was chosen for further study.

The DNA fragment encoding the 12 repeats of GnRH was subcloned into plasmid pPEDI at the 3' end of the coding region for domain Ia, the receptor-binding domain, of *Pseudomonas* exotoxin A (8, 9). The resulting plasmid, pPEDIG₁₂, was introduced into *Escherichia coli* BL21(DE3)/lysS, and the expressed fusion protein (PEIa-GnRH₁₂) was purified by taking advantage of the six-histidine (His6) tag at its NH₂ terminus (10). Affinity chromatography on a nickel-agarose column yielded a preparation of PEIa-GnRH₁₂ that was about 95% homogeneous (data not shown).

Sixteen-week-old female New Zealand White rabbits were injected three times with purified PEIa-GnRH₁₂, after which serum was individually collected for analysis. To demonstrate that the antibodies induced by PEIa-GnRH₁₂ actually reacted with the native peptide,

GnRH, reactivity of the antibody to native GnRH was measured by immunocompetitive assay using native GnRH as the competitor. As shown in Fig. 2*a*, GnRH could effectively block [³H]GnRH binding to anti-GnRH antibodies. GnRH, at equal molar concentration and 350-fold of [³H]GnRH, inhibited [³H]GnRH binding by 20 and 80%, respectively, whereas angiotensin I failed to compete for [³H]GnRH binding to anti-GnRH antibodies even at the concentration 3,700-fold to [³H]GnRH. To investigate whether the epitopes recognized by induced antibodies were localized within the GnRH sequence or spanned adjacent GnRH repeats, we examined the reactivity of the antibodies with GST fusion proteins that contained various numbers of GnRH repeats. Immunoblot analysis revealed that, although the antibodies did not recognize GST alone, they reacted (at a dilution of 1:5000) with a similar affinity to GST fusion proteins containing one to five copies of the GnRH sequence (Fig. 2*b*). In this study, we also observed that some extra bands appeared in low-molecular range. We believe that these extra bands represent the degraded products of the

fusion protein of GST and GnRH repeats. This result, thus, suggests that most of the epitopes recognized by the antibodies reside within the GnRH sequence rather than between adjacent GnRH repeats. Enzyme-linked immunosorbent assays also revealed that, at a dilution of 1:10,000, the antibodies reacted with a similar affinity to GST fusion proteins containing one or five copies of the GnRH sequence (data not shown). Thus, these observations suggest that antigens comprising linear peptide repeats can be used to generate monoclonal-like antibodies, and that these antibodies can be used for biochemical analysis of the target peptide.

To address the issue of whether multicopies or single-copy of GnRH is sufficient to induce anti-GnRH immunoresponses, we have performed animal immunization with immunogens containing various copies of GnRH. Our results showed that GnRH alone or PEIa-GnRH₁ at the dosage of 0.1 mg failed to induce an anti-GnRH immune response. However, PEIa-GnRH₉ and PEIa-GnRH₁₂ at the dosage of 0.1 mg were capable of raising high-titer anti-GnRH antibodies. PEIa-GnRH₁ at the dosage of 1 mg induced a weak anti-GnRH immune response (Fig. 3). The ELISA results of the PEIa-GnRH₁₂ group were not shown in Fig. 3, because the ELISA reading of 1,000-fold diluted anti-PEIa-GnRH₁₂ antiserum was out of scale. If the ELISA reading of the PEIa-GnRH₁₂ group were to be adjusted to the range between 1 and 2, the anti-PEIa-GnRH₁₂ antiserum needed to be diluted 10,000-fold. Under this situation, the ELISA reading of all of the other groups went down to basal levels and showed no difference from preimmune serum. This observation suggests that the copy number of GnRH in the immunogen play an important role in inducing a robust anti-GnRH immune response. In addition, the observation that PEIa-GnRH₁ at the dosage of 1.0 mg is able to induce weak immune response against GnRH, whereas GnRH at the dosage of 0.1 mg, which is in much higher molar ratio of GnRH moiety compared with 1.0 mg of PEIa-GnRH₁, fails to induce anti-GnRH immune response, clearly indicates that PEIa makes GnRH in PEIa-GnRH₁ more immunogenic. A complete study on the role of PEIa making the fusion peptides highly immunogenic is under investigation now.

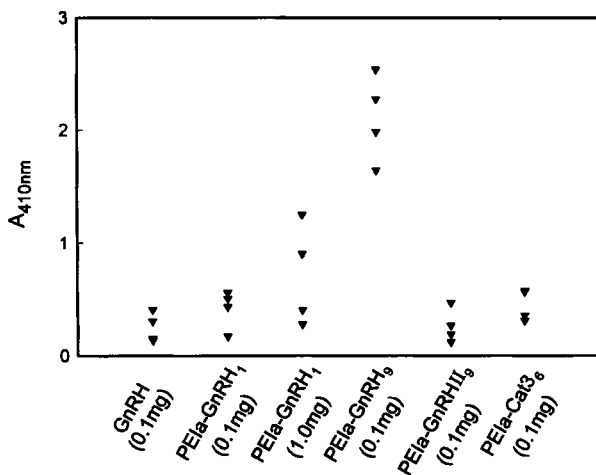


Fig. 3. Enhancement of peptide antigenicity by TR-PCR. To examine whether multicopies of peptide in linear repeat are needed to elicit peptide antigenicity, we have performed animal immunization with immunogen containing various copies of GnRH. Seven groups, four rabbits per group, of 5-month old female New Zealand White rabbits 5-month-old were injected s.c. with various dosages of immunogens three times at biweekly intervals. Serum collected from immunized rabbits (2 weeks after last immunization) was analyzed for anti-GnRH antibodies. The ELISA results were presented with 1000-fold diluted serum. The ELISA results of the PEIa-GnRH₁₂ group are not shown in this figure because the ELISA reading of 1000-fold-diluted anti-PEIa-GnRH₁₂ antiserum was out of scale. PEIa-GnRH₁₂ stands for the receptor-binding domain of *Pseudomonas* exotoxin A conjugated with nine copies of GnRH; the amino acid sequence is Gln-His-Trp-Ser-His-Gly-Trp-Tyr-Pro-Gly. PEIa-Cat36 stands for the receptor binding domain of *Pseudomonas* exotoxin A conjugated with six copies of the peptide, for which the amino acid sequence is Leu-Gly-Ile-Ser-Tyr-Gly-Arg-Lys-Lys-Arg.

a



b

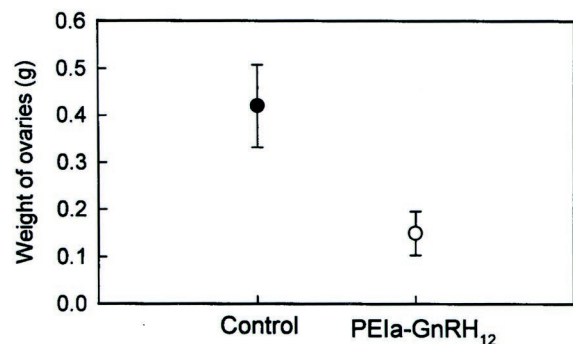


Fig. 4. Degeneration of the ovaries of rabbits immunized with PEIa-GnRH₁₂. Sixteen-week-old female New Zealand White rabbits were injected s.c. with purified PEIa-GnRH₁₂ for 3 consecutive weeks at weekly intervals. Aluminum phosphate (pH 7.0) was used as adjuvant in the injection. The injection volume was 0.5 ml and each injection contained 100 μ g PEIa-GnRH₁₂ and 0.125 mg of aluminum. a, set of control and degenerated ovaries. The ovaries of rabbits that had received three injections of either PEIa-GnRH₁₂ (left) or the control antigen PEIa-TopN₈ (right) were examined 26 weeks after the last immunization. Each experimental group contained four rabbits and all of the PEIa-GnRH₁₂ immunized rabbits showed degeneration of ovaries. The ovaries were individually weighed, statistical analysis of the control and degenerated ovaries is summarized in b.

We next investigated whether the induction of an autoimmune response to GnRH affected the ovaries of immunized rabbits. Whereas the ovaries of the four rabbits injected with a control antigen, PEIa-TopN₈ (the receptor-binding domain of *Pseudomonas* exotoxin A conjugated to eight repeats of the NH₂-terminal 10 amino acids of human DNA topoisomerase I), appeared normal, those of the four rabbits that were injected with PEIa-GnRH₁₂ exhibited substantial degeneration. One represented set of control and degenerated ovaries was shown in Fig. 4a. The ovaries were individually weighed and statistical analysis of the control and degenerated ovaries was summarized in Fig. 4b. These results indicate that the induced high-titer antibodies to GnRH appear to impair the function of this important hormone in the immunized rabbits. It has been shown that some patients with ovarian cancer have been treated with GnRH antagonist (11).

Because anti-GnRH antibody could be functionally analogous to GnRH antagonist, vaccination of PEIa-GnRH₁₂ presents a potential therapeutic application for the treatment of GnRH-sensitive ovarian cancer.

Discussion

To overcome the difficulty of inducing an immune response against poorly immunogenic self-peptides, we have designed a new and efficient delivery system for antigen presentation. In addition, we have developed a new method for increasing the copy number of the self-peptide in the antigen conjugate. We have tested our approach with GnRH as a model self-peptide. The immunogen comprised the receptor binding-domain of *Pseudomonas* exotoxin A and 12 copies of GnRH in linear alignment. This construct induced autoantibodies to GnRH that caused degeneration of the ovaries in immunized female rabbits.

In previous studies, antigens that were used to generate antibodies against small peptides have been prepared by chemical conjugation of the peptide to a carrier protein. A trial to induce antibody response to GnRH using the conjugation of GnRH to T-cell epitope has been reported (12). However, because the efficiency of such chemical conjugation varies, the antigen preparations were heterogeneous and differed from batch to batch. The immune responses to such antigen preparations were, therefore, inconsistent. With the use of our TR-PCR approach to generate a DNA template for the expression of linear peptide repeats, it is possible to synthesize large amounts of homogeneous immunogen with a high content of antigenic peptide, without the need for chemical conjugation and at lower cost. In addition to PEIa-GnRH₁₂, we have prepared several other autoantigens with the TR-PCR technique and shown that they all effectively induce an immune response (data not shown).

Our new technique may have potential applications in the development of therapeutic vaccines. Various antibodies have been shown to be effective in the treatment of cancer (13, 14). The most promising example is Herceptin, a humanized monoclonal antibody to HER2 that is now in clinical use in combination with chemotherapy (15). Vaccination with growth factor or cell surface autoantigens using our approach may offer a low-cost alternative for cancer therapy.

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